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Complementation Between Temperature-Sensitive and Deletion Mutants of Reovirus

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A very low level of complementation has been found in conventional crosses between various classes of temperature-sensitive (*ts*) mutants of reovirus. A more definitive test for complementation was devised through a plaque assay on cell monolayers mixedly infected with defective reovirions lacking the L_1 segment and prototype *ts* mutants from one or other of the known classes of reovirus mutants. An increase in the number of plaques on the mixedly infected plates over that on control plates infected with defective virions or *ts* mutants alone indicated that the *ts* mutant had been complemented by the defective virus. Class A, B, D, F, and G mutants were complemented at 39 C by the defective viruses, whereas class C and E mutants were not. In tests to determine whether complementation was reciprocal it was found that the defective virions were complemented by a class G mutant but not by the class C mutant. This and previous work (D. A. Spandidos and A. F. Graham, 1975) has therefore shown that of the seven known classes of *ts* mutants the class C mutant is the only one that neither complements nor is complemented by the defective virions. For this reason the class C *ts* mutation has been assigned to the L_1 segment of the viral genome.

A number of temperature-sensitive (*ts*) mutants of reovirus first isolated by Fields and Joklik (6) have been classified into seven groups by genetic recombination tests (4, 6). It is thought that each group of mutants represents mutations in one or another of the 10 doubled-stranded (dsRNA) segments of the viral genome and that recombination results from exchange of whole segments during replication of the two parental viral types.

Although recombination is readily demonstrable with this virus, complementation between the several mutant groups tested is very low. Thus, Fields and Joklik (6) reported that complementation indices between pairs of class A mutants and between A and B mutants were between 2 and 7. Such indices are on the borderline of significance. In a different type of test it was found (11) that in cells co-infected with pairs of dsRNA$^-$ mutants under nonpermissive conditions there was a small but significant increase in the total amount of virus-specific RNA synthesized over that of the mutants taken separately. This observation suggested a low level of complementation between C, D, and E class mutants. On the other hand we have found that defective reovirus lacking the L_1 segment is readily complemented at the nonpermissive temperature by the E class mutant, although there is no reciprocal complementation of the E function by the defective reovirus (21). This result classified the E mutation as being in a *cis*-function whereas the L_1 genomic segment codes for a *trans*-function. Consequently, lack of complementation between some mutant pairs might be explicable if several of the reoviral functions are *cis*-acting.

An opportunity to test this proposition was provided by our recent isolation of L_1-defective virions (21). In the present work we have devised a simple test with these defective virions to show that they will complement the known classes of *ts* mutants except C and E. On the other hand it is found that all *ts* mutants except the C class will complement the growth of defective virions at the nonpermissive temperature. These results suggest that of the known mutant classes only the E mutation is in a *cis*-function, whereas the C mutation is in a *trans*-function that resides in the L_1 segment of the genome.

MATERIALS AND METHODS

Cells and virus. L cells were grown in suspension in Eagle minimal essential medium supplemented with 5% fetal calf serum.

Buffers, chemicals, labeled precursors, and the conditions for growth and purification of standard (infectious) and defective virions have been described (21).

The two wild-type strains of reovirus serotype 3 previously described (21) have been used. According to the nomenclature introduced in the previous paper (21) these strains were designated R_1^3 and R_2^3

where the superscript 3 represents the viral serotype. We have also used the Lang strain of reovirus type 1 (17) (R_1^1). The following *ts* mutants of R_2^3 reovirus isolated by B. Fields and his collaborators (6) have been used as prototypes of the mutant classes: R_2^3A (201), R_2^3B (352), R_2^3C (447), R_2^3D (357), R_2^3D (585), R_2^3E (320), R_2^3F (556), R_2^3G (453). These mutants were kindly supplied by Fields. The deletion mutant R_1^3d (L_1) has also been used: this is a defective virion lacking the L_1 genomic segment whose isolation has been described (21). Permissive temperature for the plaque assay (17) was 31 C and the nonpermissive temperature was 39 C. For the present work all virus strains were freshly cloned. The wild-type strains or mutants as received from Fields were plaque titered at 31 C and an isolated plaque was picked. Each plaque was grown up to a large stock in L cells and virus from the final lysates was then purified through the standard procedure for use in the various experiments.

Standard complementation test. In this test the ability of any two mutants to complement each other was determined by co-infecting a monolayer culture (approximately 5×10^6 cells in a screw-cap bottle) with the two mutants at a multiplicity of infection (MOI) of 10 PFU/cell for each mutant. The deletion mutant R_1^3D (L_1) was used at a MOI of 200 particles/cell. Similar cell cultures were infected with each mutant separately at an MOI of 10 PFU/cell or 200 particles/cell of R_1^3d (L_1) virions. In these experiments the MOI is given as added MOI. Since the adsorption rate, measured on numerous occasions, is approximately 80% in 1 h the adsorbed MOI is 8. After 1 h at room temperature to permit adsorption of virus the cultures were washed twice with 5 ml of Eagle minimal essential medium, then 5 ml of minimal essential medium containing 2% fetal calf serum was added and the cultures were placed at 39 C. Viral growth was terminated at 18 h by freezing the cultures at −70 C. When required, the cultures were thawed, frozen and thawed three additional times, gently sonically treated for 2 min, and plaque titered at 31 and 39 C.

The complementation index for any two *ts* mutants X and Y was calculated according to the formula (6):

$$(XY_{39})^{31} - (XY_{39})^{39}/(X_{39})^{31} + (Y_{39})^{31}$$

where $(XY_{39})^{31}$ and $(XY_{39})^{39}$ are titers of the mixed yield grown at 39 but assayed at 31 and 39 C, respectively. $(X_{39})^{31}$ and $(Y_{39})^{31}$ are titers of single yields grown at 39 C and assayed at 31 C.

RESULTS

Relative plating efficiency of viral strains at 31 and 39 C. All experiments in this work were done with purified virus. Since each viral stock was developed from a single plaque and the level of complementation in this system is low it was essential to know the relative plating efficiency for each stock at 31 and 39 C. The results of such plaque assays are shown in Table 1 along with estimates of the number of particles per PFU. Each *ts* mutant differed by at least a factor of 3×10^{-4} in its plaque-forming ability at the two temperatures. The very low level of infectious virus contained in this preparation of defective virus, R_1^3d (L_1) is practically all wild type; very little of the E class mutant used to help the defective during its growth (21) is present.

Standard complementation test. Complementation tests between various mutants were carried out by the standard procedure described in Materials and Methods. Table 2 shows one example of the results of the test between class A and B mutants. The complementation index in this experiment is 5 and in a number of similar tests between the same mutants it varied between 2 and 10. In other experiments complementation between the A mutant and defective virions gave indices between 1 and 5. One set of such results is shown in Table 3 where the complementation index was found to be approximately 2.

At complementation levels as low as these it is easy to explain the variations in the results. Any one index depends on the plaque titrations

TABLE 1. *Efficiency of plating of 10 strains of purified reovirus*

Strains[a]	PFU/OD_{260} assayed at		Efficiency of plating 39 C/31 C	Particles/PFU assayed at 31 C[b]
	31 C	39 C		
R_1d (L_1)	4.7×10^5	1.2×10^5	2.6×10^{-1}	5×10^6
R_2	4.2×10^{10}	3.3×10^{10}	0.8	50
R_2A (201)	4.2×10^{10}	0.6×10^6	2.3×10^{-4}	50
R_2B (352)	3.0×10^{10}	4.2×10^5	1.4×10^{-5}	70
R_2C (447)	1.4×10^{10}	4.5×10^5	3.2×10^{-5}	150
R_2D (357)	1.6×10^{10}	2.4×10^6	1.5×10^{-4}	130
R_2D (585)	1.4×10^{10}	3.8×10^6	2.7×10^{-4}	150
R_2E (320)	2.1×10^{10}	7.0×10^5	3.3×10^{-5}	100
R_2F (556)	2.9×10^{10}	1.3×10^6	4.5×10^{-5}	70
R_2G (456)	2.6×10^{10}	1.4×10^6	5.6×10^{-5}	80

[a] All strains were cloned and clones were grown to large stocks from which virus was purified.

[b] Calculated from the ratio 1 $OD_{260} = 2.1 \times 10^{12}$ particles (20). For the defective virions R_1^3 (L_1) the ratio 1 $OD_{260} = 2.35 \times 10^{12}$ particles was used.

TABLE 2. *Virus yields after single and mixed infections with $R_2{}^3$A (201) and $R_2{}^3$ (352)*[a]

Input MOI (PFU/cell)		Incubation temp	Yield of progeny (PFU/ml) assayed at	
$R_2{}^3$A (201)	$R_2{}^3$B (352)		31 C	39 C
10	0	31	7.0×10^7	3.0×10^4
		39	5.0×10^3	1.5×10^3
0	10	31	5.0×10^7	2.0×10^4
		39	3.0×10^3	1.2×10^3
10	10	39	5.5×10^4	1.5×10^4

[a] Complementation index: $5.5 \times 10^4 - 1.5 \times 10^4/3.0 \times 10^3 + 5.0 \times 10^3 = 5$.

TABLE 3. *Virus yields after single and mixed infections with $R_2{}^3$A (201) and $R_1{}^3$d (L_1)*[a]

Input MOI		Incubation temp	Yield of progeny (PFU/ml) assayed at	
Particles/cell $R_1{}^3$d (L_1)	PFU/cell $R_2{}^3$A (201)		31 C	39 C
200	0	31	4.0×10^2	2.0×10^2
		39	1.5×10^2	1.4×10^2
0	10	31	7.0×10^7	3.0×10^4
		39	5.0×10^3	1.5×10^3
200	10	39	2.1×10^4	1.0×10^4

[a] Complementation index: $2.1 \times 10^4 - 1.0 \times 10^4/1.5 \times 10^2 + 5.0 \times 10^3 = 2$.

of three lysates with their inherent errors as well as on the viral yields from the three cultures which are low at 39 C and could vary considerably from one experiment to another. In fact, one might well question whether these complementation results taken by themselves have any real meaning. It was thought that complementation tests carried out with one member of the pair being the defective virion might be more convincing because of the very low background provided by the defective virion itself. Clearly, from the results of Table 3 this expectation was not borne out, although in some experiments with defective virions there appeared to be a low level of complementation. In searching for a more definitive and less tedious means to study complementation in this system we have devised the test described in the next section.

Plate test for complementation between ts mutants and $R_1{}^3$d (L_1) virus. This test was designed to find whether the $R_1{}^3$d (L_1)-defective virions could complement *ts* mutants. It is made possible by the observation (unpublished data) that L cells infected with these defective virions are not rapidly killed. Confluent monolayers of L cells were infected with a high MOI of defective virions along with a known number of PFU of a given *ts* mutant. After adsorption of the virus the plates were overlayed as in the standard plaque assay and incubated at 39 C, and the plaques that developed were scored. An increase in the number of plaques over the controls would represent complementation of the *ts* mutant by defective virions. A representative set of data is shown in Table 4.

To determine whether complementation occurs between a *ts* mutant and a given MOI of defective virions the total number of plaques scored at that MOI must be reduced by the number of plaques resulting from the defective virions alone and reduced again by the number of plaques given by the *ts* mutant in the absence of defective virions. The resulting number is calculated as a ratio of the number of PFU of the *ts* mutant added to the plate and is then defined as the complementation level (see

TABLE 4. *Plate test for complementation between various ts mutants and $R_1{}^3$d (L_1) virions*

ts mutants	PFU/plate added[a]	No. of plaques/plate[b]				Complementation level[d]
		0[c]	20	200	500	
R_2A (201)	10^4	3	125	Lysis	Lysis	120
	10^4	0	52	163		138
R_2B (352)	10^5	2	250	Lysis		
	10^4	0	3	27		2
R_2C (447)	10^5	4	8	28		0
R_2D (357)	10^4	2	25	98	101	71
R_2D (585)	10^4	3	21	73	84	45
R_2E (320)	10^4	0	3	26	63	1
	10^5	4	7	39	62	1
R_2F (556)	10^4	5	120	Lysis		113
R_2G (453)	10^4	0	58	161		136
None			2	25	60	

[a] PFU assay of the *ts* mutant determined at 31 C.

[b] Plates incubated at 39 C. The results represent the average number of plaques on duplicate plates.

[c] Number of particles of $R_1{}^3$d (L_1) per cell determined from OD_{260} of purified virus (see Table 1 legend).

[d] The complementation level for any coinfection between a *ts* mutant and defectives is defined as

$$\frac{\text{Number of plaques from mixed infection} - \text{number of plaques from defectives alone} - \text{number of plaques from ts mutant alone}}{\text{Number of PFU of } ts \text{ mutant plated}} \times 10^4$$

For mutants R_2A (201) and R_2F (556) the column under 20 R_1d (L_1) particles/cell was used to calculate the complementation level. For the other mutants the column 200 R_1d (L_1) particles/cell was used.

legend to Table 4). Essentially the complementation level as defined represents those cells, a very small proportion of mixedly infected cells in fact, in which complementation is so efficient that the progeny virus develops a plaque despite the nonpermissive temperature. Complementation probably occurs in other mixedly infected cells as well but at too low a level to be perpetuated into a plaque. Complementation was measured in this test and not recombination, because, as we will show in a later paper, there is no detectable recombination between these defective virions and any known class of *ts* mutants.

A complementation level as defined in Table 4 can be calculated for each mutant at each of the three MOIs of defective particles. When this is done it is found that complementation is most efficient at an MOI of 200 R_1^3 (L_1) particles/cell (Table 4). In two cases, R_2A and R_2F, lysis was almost complete on the plates, and the complementation levels have been computed on the results obtained with an MOI of 20 defective virions/cell.

It is clear that the E mutant is not complemented by any MOI of defectives used (column 7, Table 4). This is the expected result for this mutant because it has already been shown in a different way that the E mutant is not complemented by defectives and that the E *ts* mutation is in a *cis*-function (21). Thus, the result with the E mutant provides a base line for comparison (Table 4). Against this base line the A, B, F, and G mutants are clearly complemented. The level of complementation of the two D mutants is lower but well above the base line. It can be calculated that approximately 1.4% of the cells infected with 10^4 PFU/cell of B mutant show complementation in this test (column 5, Table 4). For the two D mutants 0.5 to 0.7% of the cells infected with 10^4 PFU/ml showed complementation. On the other hand, the C mutant was not complemented and this result defines the C mutation to be either in a *cis*-function or in the L_1 segment of the genome.

Complementation of defective virions by ts mutants. Previously we showed that when cells were co-infected with defective virions and class A, B, or E *ts* mutants at 39 C there was extensive growth of the defective virions (21). All three mutants had complemented the missing L_1 function. Indeed it would be predicted that any *ts* mutant would complement this defective virion unless it had the mutation in the L_1 genomic segment, and the results of Table 4 suggested that the class C mutant might be in this category. The following experiment was therefore carried out to determine whether the C mutant would complement the growth of defective virions using as controls the wild-type virus and G mutant.

Three suspension cultures were co-infected with defective virions and one or another of wild-type virus, G mutant, or C mutant and placed at 39 C. A second set of three cultures was infected in the same way and placed at 31 C. The same amount of [^{3}H]uridine was added, with actinomycin D, to each culture to label the progeny virus. When the cells had partially lysed, the cultures were centrifuged and a known amount of ^{14}C-labeled, purified wild-type virus (which contained no defective virions) was added to each sediment. This ^{14}C-labeled virus was to act as a carrier, a marker to measure the efficiency of each purification step, and subsequently as a sedimentation marker in gradient analyses. Virus from each sediment was purified and the recovery of ^{3}H and ^{14}C at each step was determined. From the respective value for recovery of added ^{14}C-labeled virus and the amount of material with an optical density at 260 nm (OD_{260}) in each purified preparation the yield of viral particles per cell was calculated for the various cultures. The results are shown in Table 5.

Overall recovery of added ^{14}C-labeled virus varied from 70 to 80%. The co-infection with wild-type virus and R_1d (L_1) at 31 C gave a normal yield of particles per cell (21), whereas the yields with the two *ts* mutants were considerably lower; these have been consistent observations. The yields of particles per cell were all considerably less at 39 C than at 31 C and, in fact, no virus particles were synthesized in the co-infection between the C mutant and R_1d (L_1) virions at 39 C consistent with the view that the class C *ts* mutation resides in the L_1 segment.

To determine the nature of the particles synthesized, the final purified virus from each of the six cultures was digested with chymotrypsin to convert the virions to cores (12, 15) and the products were centrifuged to equilibrium in CsCl gradients. The results for the 31 and 39 C cultures are shown in Fig. 1 and 2, respectively.

The leading peak of ^{3}H in each gradient of Fig. 1 and 2 (ρ = 1.43 g/ml) coincides with the ^{14}C peak of the marker and represents cores derived from infectious virions, either wild-type or *ts* mutant. The trailing peak at ρ = 1.415 g/ml represents cores derived from defective virions (14, 15, 21). Since the relative amounts of cores in each gradient represents the relative amounts of standard and defective virions in the purified virus, it is clear from Fig. 1 that at 31 C roughly equal amounts of infectious and defective virions were synthesized in each of the three cultures. Because defective virions

TABLE 5. *Yields of purified virus from cultures doubly infected with defective virions and wild-type or mutant virions*[a]

Infection	Recovery of ^{14}C-labeled virus[b] (%)	Purified virus recovered[c] particles/cultures × 10^{12}	Yield of virus[d] particles/cell × 10^4
31 C			
$R_2 \times R_1d$ (L_1)	78	8.7	8.8
$R_2C \times R_1d$ (L_1)	73	3.1	1.9
$R_2G \times R_1d$ (L_1)	81	4.0	2.6
39 C			
$R_2 \times R_1d$ (L_1)	76	3.3	2.0
$R_2C \times R_1d$ (L_1)	81	1.9	0
$R_2G \times R_1d$ (L_1)	70	2.6	1.4

[a] The general method of carrying out the experiment is described in the text. Each culture contained 200 ml at 5 × 10^5 cells/ml coinfected with 500 particles per cell of $R_1{}^3d$ (L_1) virions and 10 PFU/cell of one or other of the viruses, $R_2{}^3$, $R_2{}^3C$ (447), $R_2{}^3G$ (453); 0.5 μg of actinomycin D/m l; 1 μCi of [^{3}H]uridine/ml. After 40 h at 31 C or 12 h at 39 C the cells were centrifuged and to the sediment was added 2.35 × 10^{12} particles of purified $R_2{}^3$ virus containing 8 × 10^4 counts/min of ^{14}C in its RNA. Purification of virus from the sediment followed the previously described method (21).

[b] Total ^{14}C-labeled virus recovered after purification as a percentage of that added before purification.

[c] Total purified virus recovered from each culture including the added ^{14}C-labeled virus. Particles per culture calculated from the relationship, 2.35 × 10^{12} particles/OD_{260}, see footnote Table 1.

[d] Calculated from the results in the preceding column by correcting the figures for recovery of ^{14}C-labeled virus and then subtracting the number of ^{14}C-labeled particles originally added.

cannot replicate alone, nor synthesize progeny dsRNA (unpublished data), growth of the defective virions has been "helped" at 31 C by the presence of infectious virions. A similar helping effect has been shown with the A, B, and E mutants at 31 C (21).

A different situation is seen at 39 C (Fig. 2). Although the overall yield of particles was considerably reduced (Table 5), wild-type virus at 39 C helped (complemented) the defective virions as efficiently as at 31 C. Most of the virus formed in the co-infection of G mutant and R_1d (L_1) virions at 39 C was defective: the G mutant had efficiently complemented growth of the defective virion, although there was limited reciprocal complementation. In a control culture infected with G mutant alone (results not shown) there was practically no growth of the mutant at 39 C and no defective virions were formed. This control shows that the defective virions did not arise from the *ts* mutant itself as has also been found with the A and B mutants (21). The middle panel of Fig. 2 shows that there was no growth of any virus at 39 C in the co-infection between C mutant and defective virions.

These results are interpreted to mean that the G mutant can readily complement the growth of defective virions at 39 C but is itself poorly complemented by the defective virion. Similar remarks applied to co-infections between the defective virus and the A or B mutants (21) and also to the D and F mutants (unpublished data). The low level of growth of A, B, D, F, and G mutants at 39 C in this type of experiment is consistent with the rather low level of complementation of these *ts* mutants by defective virions shown in Table 4. In contrast, there is no complementation in either direction between defective virions and the C mutant at 39 C, although both virions grow reasonably well together at 31 C.

Complementation of $R_1{}^3d$ (L_1) virions by reovirus type 1. Whereas types 1 and 3 reoviruses are serologically distinct, the sizes of the three L segments of the two serotypes are similar. It was therefore of interest to determine whether the type 1 virus would complement the type 3-defective virions. A culture of L cells was co-infected with $R_1{}^1$ virus and $R_1{}^3d$ (L_1) virions at 31 C and the viral progeny was labeled with [^{3}H]uridine during its growth. After purification ^{14}C-labeled $R_2{}^3$ virus was added as a marker and the resulting mixture was digested with chymotrypsin to convert it to cores and subjected to isopycnic centrifugation in CsCl (Fig. 3a). In a control experiment a culture was infected with type 1 virus alone and subjected to similar manipulations. The results of the control experiment (Fig. 3b) show that infection with type 1 virus alone did not give rise to defective virus. However, the gradient of Fig. 3a shows a considerable peak at the buoyant density of defective cores (ρ = 1.415 g/ml) that resulted from the mixed infection. Thus type 1 virus can readily help the growth of the type 3-defective virions. We have recently suggested that the L_1 segment of the viral genome codes for the λ_2 polypeptide of the viral core (13). If this is so the defective cores, resulting from the co-infection of $R_1{}^1$ virus and $R_1{}^3d$ (L_1) virions, should be a phenotypic mixture containing the λ_2 polypeptide of type 1 virus with the remaining core peptides, λ_1, μ_1, and σ_2 (20), being type 3 in origin. Experiments are in progress to test this prediction.

DISCUSSION

The use of defective virions in this study has served two main purposes: first, to demonstrate unequivocately that complementation in the

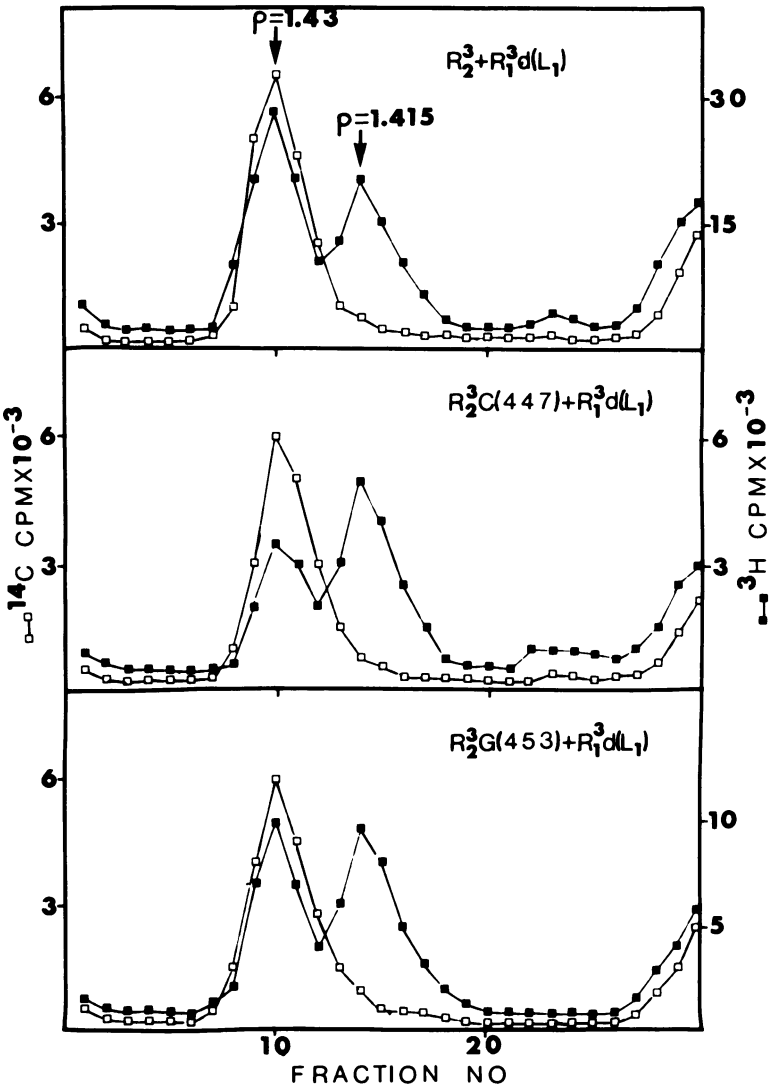

FIG. 1. *Cesium chloride gradient analysis of the viral cores obtained from the 31 C progeny of co-infection between defective virions and wild-type or ts virus. The purified viral yields from the 31 C cultures described in Table 5, containing ^{14}C-labeled marker R_2^3 virus, were dialyzed against 0.05 M Tris-hydrochloride buffer, pH 8.0, and digested for 60 min with 100 μg of chymotrypsin per ml. The resulting mixture was layered over a preformed gradient of CsCl (ρ = 1.35 − 1.46 g/ml) and centrifuged for 6 h at 37,500 rpm in the Beckman SW40 rotor. Fractions were collected from the bottom of the punctured tube and analyzed for density and radioactivity. The particular cross used in each coinfection is described in the appropriate panel. Symbols: (□) ^{14}C-labeled marker virus; (■) ^{3}H-labeled progeny of the co-infection.*

reovirus system can be recognized by genetic techniques and, second, to allocate the class C mutation to the L_1 segment of the viral genome.

Levels of complementation in the standard test were too low and variable to be sure of their significance. However, we have devised a much more sensitive test for complementation in which cell monolayers were first spread with defective virions, then superinfected with a *ts* mutant and overlayed. In some of these crosses a significant fraction of the doubly infected cells gave rise to plaques (Table 4). Although no recombination has been detected between R_1d (L_1) virions and any class of *ts* mutants (unpublished data), there is still a possibility that some co-infected cells might give rise to ts^+ recombinants. Even so, a preliminary complementation would have to occur between the parental virions to provide the genomes to recombine with each other. Whatever the mecha-

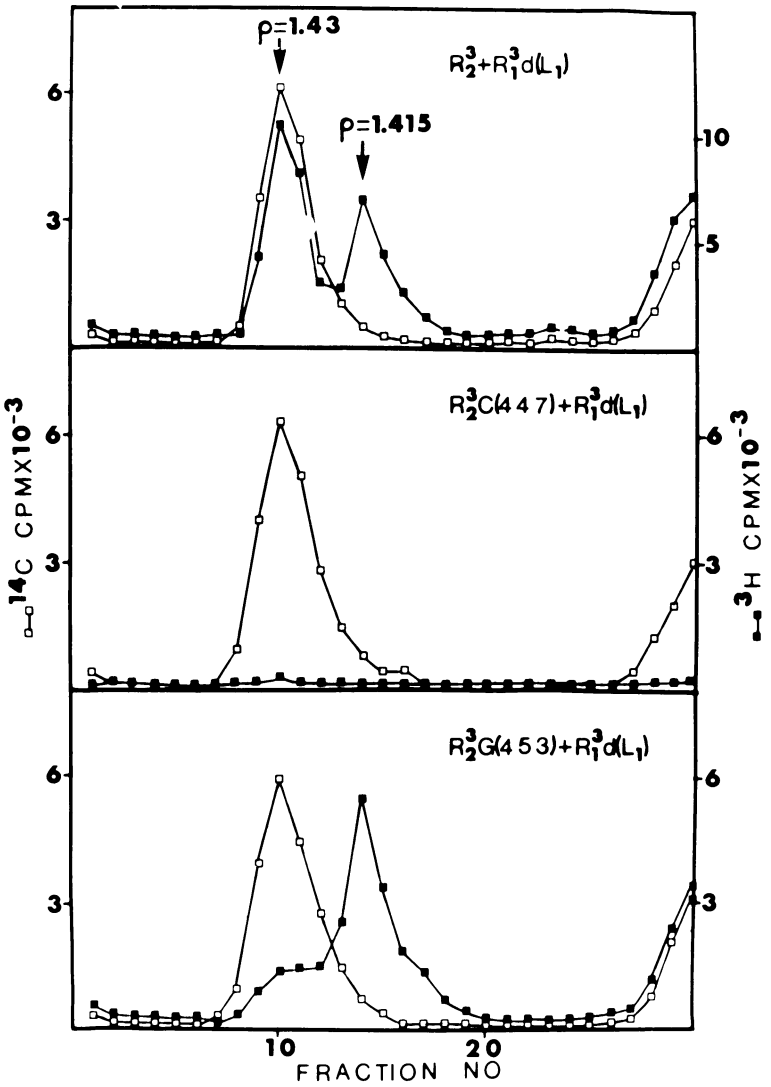

FIG. 2. *Cesium chloride gradient analysis of the viral cores obtained from the 39 C progeny of co-infection between defective virions and wild-type or ts virus. The purified viral yields from the 39 C cultures described in Table 5 were treated as described in the legend to Fig. 1. Symbols:* (□) ^{14}C*-labeled marker virus;* (■) ^{3}H*-labeled progeny of the co-infection.*

nism of plaque development in this test the primary event must be complementation. Thus, all known classes of *ts* mutants except C and E are complemented by R_1d (L_1) virions at the nonpermissive temperature.

Conversely, growth of defective virus at the nonpermissive temperature is clearly complemented by wild-type virus and the class G mutant (Fig. 2), by the A, B, and E mutants (21), by D and F mutants (unpublished data), and even by serotype 1 reovirus (Fig. 3). The class C mutant is the only one that neither complements defective virions nor is complemented by them. Thus, with some confidence we can assign the class C *ts* mutation to the L_1 segment of the viral genome.

One practical upshot of this work may be in the use of R_1d (L_1) virions to select new *ts* mutants that are not complemented by the defective virions. Seven different classes of *ts* mutants are known and, since each class is presently thought to represent a different segment of the genome, there should be three classes remaining to be discovered. Moreover, only one mutant in each of classes C and E is known (4) and it would be useful to have more. The plate

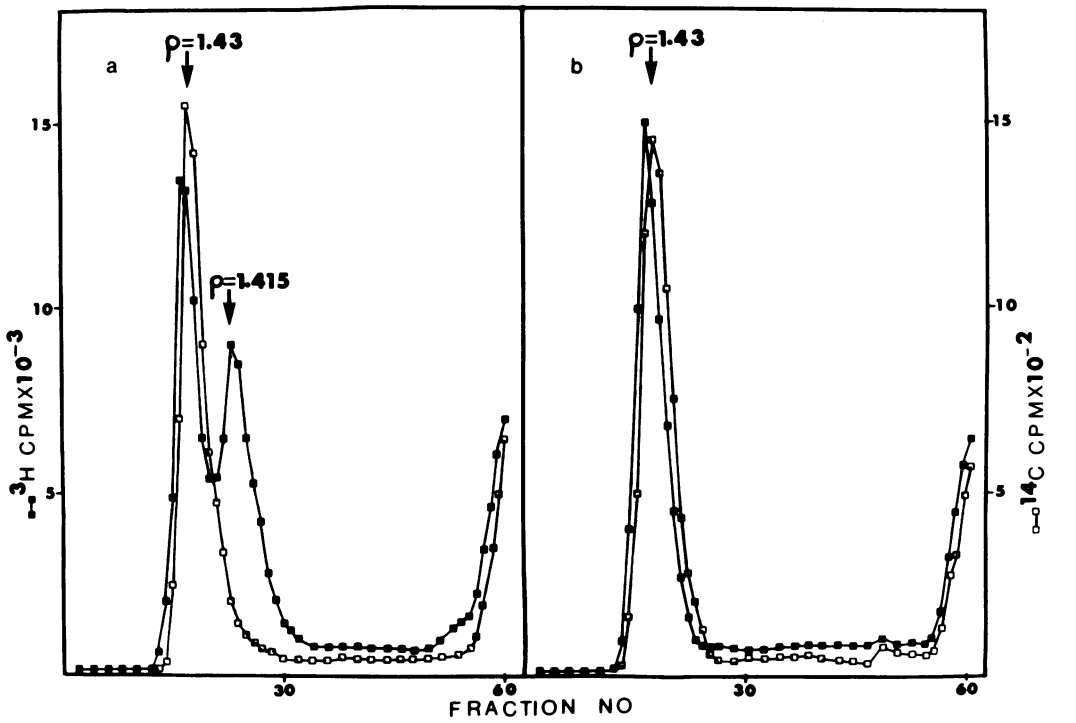

FIG. 3. *Cesium chloride gradient analysis of viral cores obtained from the co-infection between defective type 3 virions and type 1 reovirus. A culture containing 200 ml at 5 × 10^5 L cells/ml was co-infected with 2,000 $R_1{}^3d$ (L_1) virions and 10 PFU of $R_1{}^1$ virus/cell, and 0.5 μg of antinomycin D per ml and 10 μCi of [^{3}H]uridine/ml were added. A second culture was treated in the same way after infection with 10 PFU/cell of $R_1{}^1$ virus. The progeny virus was purified, mixed with purified ^{14}C-labeled $R_2{}^3$ virus as a marker and treated as described in the legend to Fig. 1. (a) Co-infection with $R_2{}^3d$ (L_1) and $R_1{}^1$ virions; (b) infection with $R_1{}^1$ virus. (□) ^{14}C-labeled marker virus; (■) ^{3}H-labeled progeny of the infection.*

method described in Table 4 could be used to test newly isolated *ts* mutants for complementation by R_1d (L_1) virions. Those *ts* mutants not complemented in the test would be class C or E or a new class. In fact, one might predict that at least one new class of mutants would be isolated in such a test, namely those with *ts* mutations in the virion transcriptase (1, 19), which should be a *cis*-acting function. Although it has been suggested that the virion transcriptase of the class G mutant is slightly *ts* (2) it is shown that this mutant is complemented by R_1d (L_1) virus (Table 4) and that it complements the defective virion (Fig. 2). Consequently the major mutation in the G class must be elsewhere than in the transcriptase. There is no evidence that either the C or E mutation affects the virion transcriptase although both mutants are dsRNA$^-$ (2, 11). In fact, the evidence is to the contrary in that both genomes have been shown to be transcribed in infected cells under nonpermissive conditions (2, 11) and such transcription must be carried out by the parental virion polymerase. As will be described in detail elsewhere the genome of R_1d (L_1) virions is also transcribed in vivo, although no dsRNA is formed.

Although the plate test with R_1d (L_1) virions shows that they will complement various *ts* mutants, it does not provide a method for classifying such mutants into complementing groups. The plate test, taken in conjunction with the results of the reciprocal test (e.g., Fig. 1 and 2), has nevertheless specified that the A, B, C, D, F, and G *ts* mutations are in *trans*-functions, whereas the E mutation is in a *cis*-function. Complementation should therefore occur between *ts* mutants of reovirus in different *trans*-acting functions. This encourages one to proceed with a systematic application of the standard complementation test to the reovirus mutants. Even though complementation indices may be of borderline significance in some crosses, it is important to find whether the classification of mutants according to their functional differences corresponds to that based on recombination frequencies (4, 6).

Pure populations of different deletion mu-

tants will be needed to allocate other *ts* mutant classes to their specific segments by the complementation plate test described here. Since six of the seven known viral functions are *trans*-acting any one of these could theoretically be deleted. In fact, on continued passage of practically all strains of reovirus, wild type or *ts*, a spectrum of defectives is obtained (unpublished data) some with double deletions of whole segments and at least one with a partial deletion. Some *ts* mutants, for example the class C mutant, generate defectives with high frequency even at low multiplicities of infection (18). Isolation of specific defective virions from such mixtures, in which virions with L_1 deletions are usually in large excess, is a formidable technical problem for which there is no apparent general solution. On the other hand production of defective viruses may well modify the course of an infection with the parental strain as has been discussed by Huang and Baltimore (10). Evidence is accumulating that the development of persistent viral infection both in cell cultures and in animals frequently involves the selection of *ts* mutants during the infection (16). In fact subacute neurotropic infections in mice have been induced through exposure of the animals to certain *ts* mutants of reovirus (5, 7) and of measles virus (8). Development and maintenance of these chronic infections could be simply explained by the long-term limited growth of the *ts* mutants under temperature conditions very close to nonpermissive. It is also possible that some subacute viral infections may be the result of a complex interplay between a *ts* mutant and defective virions generated from it or from the parental wild-type strains, as has been shown for persistent infections with vesicular stomatitis virus for example (3, 9). Continued analyses of the sort presented in this paper should give some insight into how such mixed viral populations can maintain themselves through mutual complementation.

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